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FLUORESCENCE SPECTROSCOPY OF PLANAR BLACK LIPID MEMBRANES

PROBE ADSORPTION AND QUANTUM YIELD DETERMINATION

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SUMMARY

A technique is described for the examination of fluorescence in planar black lipid membranes. Special attention has been devoted to the elimination of light scattered from the border regions of the membrane and from the numerous small lenses of hydrocarbon solvent which can form in certain types of black film. Fluorescence spectra of 1-anilino-8-naphthalene sulphonate (ANS) are reported for phospholipid and monoglyceride membranes. The adsorption of the fluorescent probe has been measured by a thermodynamic method and the quantum yield of the probe in the membrane has been determined. Both the emission spectrum and the quantum yield of ANS in black films agree well with the respective data obtained from measurements on sonicated aqueous dispersions of the lipid.

INTRODUCTION

The application of fluorescence spectroscopy to the study of structure in biological membranes has developed considerably during the past few years[1]. Concomitantly, it has become desirable to understand the properties of fluorescent molecules in membranes of known composition and structure. As a consequence, spectroscopic studies of fluorescent probe molecules have been reported for lamellar liquid crystalline dispersions (liposomes)[2], lamellar lipid–water phases[3] of phospholipids and for spherical black films[4]. For many purposes, however, these membranes are not very convenient in that either transport processes may be difficult to measure accurately, or the range of lipids, for which stable membranes are formed may be too restricted. In these respects, the planar black lipid membrane has definite advantages but, as has been found by Yguerabide and Stryer[4] and others[5–7], this approach is beset with some awkward technical difficulties. The first of these is that the use of conveniently small black films gives rise all too easily to interference by light scattered from the border

Abbreviations: ANS, 1-anilino-8-naphthalene sulphonate; PTFE, polytetrafluoroethylene.

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regions. Obviously, it is possible to circumvent this problem by using large films, an approach which allows the use of standard fluorescence instrumentation with only slight modifications[5,6,8]. This technique, however, has the serious disadvantage that it is difficult to judge the optical quality of a black film under the low magnification employed. It is known that numerous, often very small lenses of bulk liquid[9], which become trapped in the more well defined types of black film[10,11], can give rise to a considerable amount of scattered light. This scattering may be prohibitively large if reproducible quantitative measurements are desired for low membrane concentrations of the probe molecules. If the fluorescent probe molecule is water soluble, the fluorescence of the aqueous phase has to be taken into account, its amount being sensitive to scattered excitation light.

A further serious problem for quantitative studies is the necessity to establish the number of probe molecules in the membrane. This would require either the knowledge of their quantum yield or the assessment of the membrane composition by an independent method.

The main purpose of the present paper is to report an investigation in which these difficulties have been overcome. The technique will be described in some detail and its use is illustrated by the measurement of the fluorescence spectrum of a water soluble probe molecule, 1-anilino-8-naphthalene sulfonate (ANS), in membranes of egg yolk phosphatidylcholine and of glyceryl monooleate. In addition, the adsorption of the ANS to the glyceride membrane has been measured by a thermodynamic method and the quantum yield for the ANS in the membrane determined.

MATERIALS AND METHODS

Materials

The glyceryl monooleate was obtained partly from Sigma and partly it was kindly synthesized and provided by Dr H. Eibl (Max-Planck Institut für biophysikalische Chemie, Göttingen). The egg yolk phosphatidylcholine was kindly supplied by Mr N. Miller of the Agricultural Research Council (Institute of Animal Physiology, Babraham). *n*-Decane was of Puriss. grade from Koch-Light and was further purified by passage through an alumina column. NaCl was of A.R. grade and was roasted at 700 °C to remove organic impurities. The sodium salt of 1-anilino-8-naphthalene sulfonic acid (ANS) was originally a technical grade specimen from Eastman. It was further purified by dissolving in methanol, filtering, evaporating to near dryness and adding benzene, whereupon the ANS was precipitated. The precipitate was filtered off and washed with benzene and diethyl ether. The same procedure was then repeated using acetone instead of methanol and followed by precipitation from acetone with diethyl ether. The final product was a pale yellow powder which at $\leqq 10^{-3}$ M in water gave a molar extinction coefficient of $(4.9 \pm 0.25) \cdot 10^3$ mole$^{-1} \cdot$cm^{-1} at 360 nm. Water was twice distilled, first from a commercial still and then from a Pyrex vessel fitted with a quartz column, condenser and receiver.

The membranes

Horizontal black lipid membranes were formed in a cell shown schematically in Fig. 1. The hole in the polytetrafluoroethylene (PTFE) support, across which the film was extended, had a diameter of approx. 2.5 mm. The filling of the hole was accom-

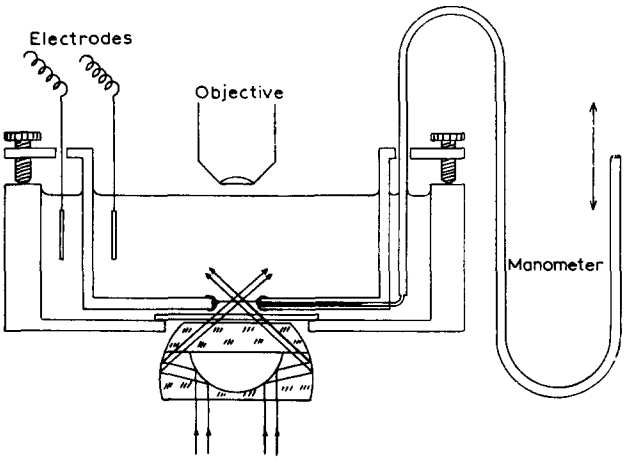

Fig. 1. Schematic drawing of the black film cell. The horizontal black film is illuminated by a darkground condenser through a thin window in the outer cell, and is viewed by means of a microscope, the objective of which is shown.

plished by means of a manometer or syringe, as described elsewhere[12]. Owing to the relatively large diameter of the hole, the lipid solution tended to escape during filling and gravitated to the surface of the aqueous solution. This was avoided by the temporary insertion of a small PTFE rod. Once the hole was filled the rod could be removed. Measurements of the membrane resistance and capacitance were carried out as in earlier investigations[13]. Interfacial tensions were determined by the drop-volume technique[14], the bulk hydrocarbon phase being pre-equilibrated with the initial aqueous solution. All experiments were performed at 20 ± 1 °C.

Aqueous sonicated dispersions

Aqueous dispersions were prepared by sonication of the lipids at nominal lipid concentrations of approx. 10^{-3} M and subsequent dilution with the appropriate solutions. The glyceryl monooleate dispersions were not stable in 0.1 M NaCl and even in the absence of salt a small amount of ANS had to be added to increase the surface charge of the dispersed particles and prevent flocculation. Therefore, the glyceryl monooleate dispersions were prepared in a $2 \cdot 10^{-4}$ M NaCl solution which was $2 \cdot 10^{-5}$ M with respect to ANS.

The spectrophotometers

The molar extinction coefficient of ANS was obtained by means of a Pye Unicam SP 800 or a Beckman DK-2A spectrophotometer. The absorbance data for the corrections of the fluorescence measurements on sonicated dispersions were determined with a Cary 14 differential spectrophotometer. The fluorescence data on the dispersions were obtained with a Fica 55 (Société française d'instruments de contrôle et d'analyses), an instrument allowing the recording of fluorescence spectra which are fully corrected both for variations in the excitation light intensity (Rhodamine B quantum counter) and for the wavelength sensitivity of the emission monochromator and photomultiplier. The slits on both monochromators were chosen to give band widths of 7.5 nm.

The microfluorimeter: optics

The microfluorimeter was essentially a fluorescence microscope (Reichert Zetopan), suitably modified by the use of quartz lenses in the illumination path, and by the addition of a graded strip interference filter and a photomultiplier in order to analyse the fluorescence radiation (Fig. 2).

The incident light was provided by a 100-W tungsten halogen lamp (L) powered by a stabilised dc source. The light was filtered (Reichert UG 1 filter, $\lambda_{max}=360$ nm, half width 60 nm [F_1]) and a heat filter (Zeiss KG 1 [F_2]) and focused on to the horizontal black film by means of an immersion dark ground condenser. The use of the latter made it necessary for the distance between the front face of the condenser and the membrane to be very small (approx. 0.5 mm). This was achieved by cementing a thin (0.1 mm) glass window into the bottom of the outer (perspex) vessel. Crude adjustments of the position of the black film were made through levelling the inner vessel by means of three screws. To bring the black film into the position of maximum light intensity, fine adjustments could be carried out by the addition or removal of small amounts of aqueous phase from one or other of the two cell compartments so that the hydrostatic pressure difference moved the film vertically in the hole without causing bulging of the film. The illuminated area was always smaller than that of the black film itself, and thus only scattered ultraviolet light could excite fluorescence in the torus.

Visual observation of the film was achieved by means of light from the opaque illuminator (OI), reflected from the film into the binocular viewing head (P_1, E_1). To remove glare caused by internal reflections in the microscope, an annular aperture diaphragm (Stach diaphragm [AD]) was fitted.

The selection of the objective was determined by a compromise between the

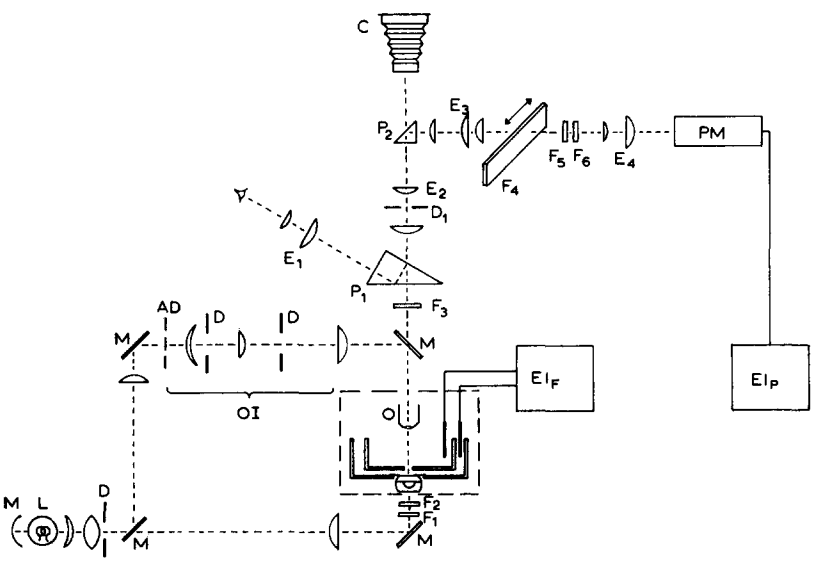

Fig. 2. Schematic representation of the whole apparatus. L, light source; M, mirror; D, iris diaphragm; AD, annular diaphragm; F, filter; E, eyepiece; P, prism; C, camera; PM, photomultiplier; O, objective; EI_F, black film electronics; EI_P, photometric electronics; OI, opaque illuminator. (See text for full description.)

requirements of, on the one hand, a sufficiently low magnification and, on the other, a high numerical aperture (light intensity). For most measurements a Reichert Epi 5.5/0.15 objective (working distance approx. 18 mm) was used without immersing it in the aqueous phase. As the ratio of scattered light to useful fluorescent radiation increases with higher numerical apertures, the relatively small aperture (0.15) of this objective was no disadvantage in terms of signal to noise ratio. In cases where a higher magnification (and resolution) is required either an Epi 11/0.25 (with or without immersion) or an Epi 28/0.55 (immersed, Epi condenser removed) may be used.

After passing the objective, the light was filtered (Zeiss 467867 [F_3]) to remove red light transmitted by the excitation filter (F_1). An eyepiece (6.3) (E_2) in the vertical phototube of the microscope, fitted with an iris diaphragm (D_i) (located in the image plane), allowed selected portions of the image to be measured. Thus, any fluorescence of the black film torus could easily be blocked out. A movable beam splitter (P_2), which was part of the regular camera system (Reichert Kam ES [C]), directed either 100% (no light reaches the camera) or 20% (80% reaches the camera) of the light intensity into the photomultiplier side-arm. The latter was attached in place of the monocular eyepiece which is normally part of the camera system. By means of auxiliary lenses (E_3, E_4) (taken from regular 6.3 x and 8 x eye pieces) the light was focused to a small spot in the plane of the graded strip interference filter (F_4) (Barr and Stroud SGS 2). The light passed two additional filters (Zeiss EG 38 [F_5]; yellow filter, Zeiss GG 3 [F_6]) and a mechanical shutter before being projected on to the cathode of the photomultiplier (EMI 9558 B). Generally, when selecting colour glasses as auxiliary filters, care has to be taken to avoid filter fluorescence. Some types of filters even show prohibitively strong phosphorescence. The filters chosen showed satisfactory performance under the conditions of this study, so that the effects of filter fluorescence could be neglected.

The relative spectral sensitivities of the total light collecting path were determined. These data take care of filter and other transmission losses, and the spectral response of the photomultiplier. They allow the correction of the recorded emission spectra. Thus, the ordinate scale in the corrected spectra gives the values of the photocurrent which would have been obtained if the sensitivity of the instrument were unity. The spectral bandwidth of the instrument was approx. 13 nm.

The microfluorimeter: electronics

The photomultiplier was operated at a gain of 200 A/lm. The dark current was approx. 0.5 nA. A linear dynode chain was used (100 kΩ resistors). The photocurrent was obtained by measuring the voltage drop across a 1-MΩ anode resistor, the time constant of the photomultiplier circuitry being approx. 0.5 s.

The position of the graded strip interference filter was linearly related to the wavelength of the transmitted light. To facilitate the recording of the uncorrected spectra the position of the filter was sensed by a helipot used as a voltage divider. Thus, a wavelength signal could be fed into the X-axis of an X–Y recorder. The photomultiplier signal was fed into the Y-axis.

RESULTS

General

Black lipid membranes were formed either from glyceryl monooleate or from

egg yolk phosphatidylcholine in solutions in decane. The aqueous phases consisted of 0.1 M NaCl and ANS in concentrations up to $2 \cdot 10^{-4}$ M.

The ANS did not affect the stability of the black membranes but it did produce an increase in the specific conductance of the membrane (for 50 mV applied), typically from $< 10^{-9} \Omega^{-1} \cdot cm^{-2}$ in absence of ANS to approx. $3 \cdot 10^{-8} \Omega^{-1} \cdot cm^{-2}$ at $5 \cdot 10^{-5}$ M ANS. These values were reproducible only to within $\pm$ 50%. No significant change in the membrane specific capacitance ($0.389\ \mu F \cdot cm^{-2}$) was detected.

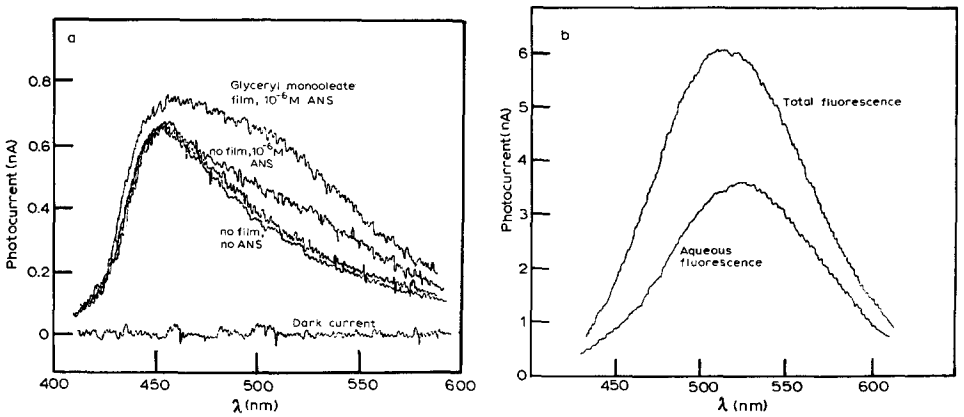

Fig. 3. Typical uncorrected spectra, traced directly from the X–Y recorder curves to give a qualitative indication of the signal to noise ratio. (a) Without ANS and with 10^{-6} M ANS in the aqueous phase, (b) With $3 \cdot 10^{-5}$ M ANS in the aqueous phase.

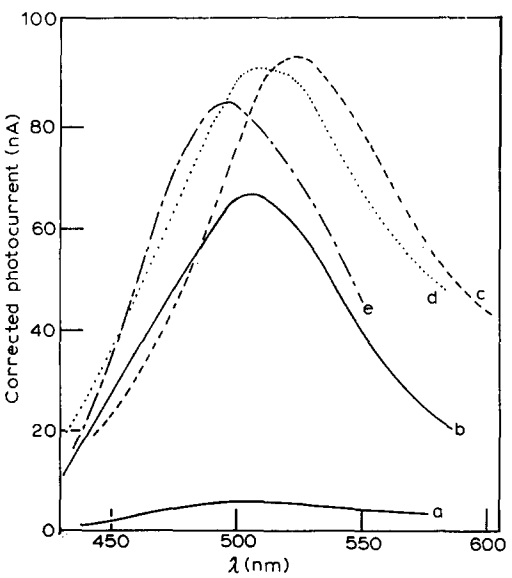

Fig. 4. Fluorescence spectra corrected for the wavelength sensitivity of the apparatus. (a) Fluorescence of a black glycerol monooleate membrane in the presence of 10^{-6} M ANS, $\lambda_{max} = 505$ nm. (b) As (a) but for $3 \cdot 10^{-5}$ M ANS; $\lambda_{max} = 505$ nm. (c) Aqueous phase fluorescence for $3 \cdot 10^{-5}$ M ANS; $\lambda_{max} = 525$ nm. (d) Aqueous phase fluorescence after rupturing an egg phosphatidylcholine membrane. Note the shift in the emission peak. ANS concentration $3 \cdot 10^{-5}$ M. (e) Fluorescence of a black egg phosphatidylcholine membrane for $3 \cdot 10^{-5}$ M ANS; $\lambda_{max} = 492$ nm.

Emission spectra

Emission spectra of ANS adsorbed on to black films (film spectra) were obtained by subtraction of the spectrum of ANS in the aqueous phase when no membrane was present (aqueous phase spectrum) from the spectrum when a membrane was present (total spectrum). Some examples of such spectra (uncorrected for the wavelength sensitivity of the instrument) are shown in Fig. 3. This procedure is valid only under certain conditions, which will be explained in the appendix, together with a detailed description of the necessary control experiments.

Spectra (corrected for the wavelength sensitivity of the instrument) for ANS in the aqueous phase and in black lipid membranes are shown in Fig. 4. For black films of glyceryl monooleate the maximum of the ANS emission spectrum is shifted 20 nm to 505 ± 2 nm while for egg phosphatidylcholine the shift is 33 nm to 492 ± 2 nm. The fluorescence of ANS in the aqueous phase showed an emission maximum at 525 ± 5 nm. The position of the maxima did not depend on the aqueous ANS concentration over the range studied.

Determination of the quantum yield of ANS adsorbed on to black lipid membranes

The quantum yield was determined relative to the known quantum yield for the ANS in water[15]. Owing to the complicated illumination geometry (Fig. 5) a special but simple procedure was adopted. The following definitions and relations are necessary.

The integrated fluorescence intensity, F, is obtained from the photocurrent $I(\lambda)$, caused by fluorescent light at wavelength λ, by the relations

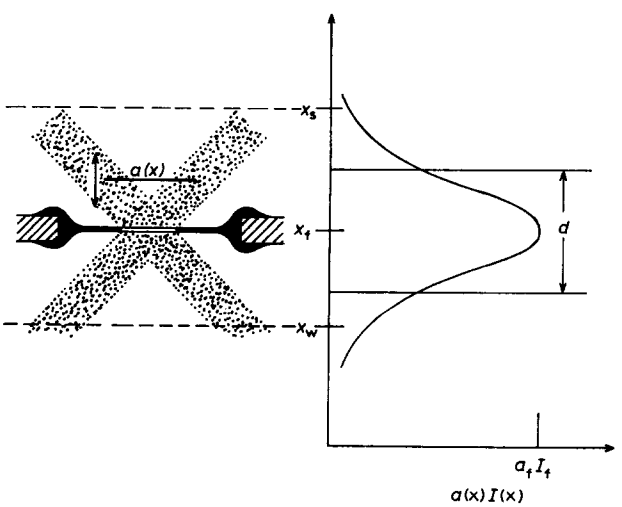

Fig. 5. Schematic representation of the illumination geometry used in the present technique. A small area a_f of the black film is illuminated by a hollow cone of light. The effective illuminated volume of the aqueous phase is determined by the shape of this cone and the area A of the measured field, as limited by the image field diaphragm. x_w, x_f, x_s are the positions of the window, film and aqueous phase surface, respectively. $I(x)$ is the light intensity (per cm^2) as a function of x, $a(x)$ $I(x)$ being the total measurable light intensity. d is the equivalent illuminated layer thickness.

$$F_f = \alpha \int i_f(\lambda) d\lambda = i_{f,max} S_f \quad (1)$$

and

$$F_o = \alpha \int i_o(\lambda) d\lambda = i_{o,max} S_o \quad (2)$$

The subscripts f and o refer, respectively, to the black film and the aqueous phase. α is an instrumental constant. The terms $i_{f,max}$ and $i_{o,max}$ are the corrected photocurrents corresponding to the wavelength of maximum fluorescence intensity, and S_f and F_o are related to the quantum yields ϕ_f and ϕ_o in the film and aqueous phase by the definitions

$$F_f = a_f I_f N_f \Phi_f \quad (3)$$

and

$$F = N_o \cdot \Phi_o \int_{x_w}^{x_s} I_o(x) a_o(x) dx = N_o \Phi_o L \quad (4)$$

where I_f and $I_o(x)$ are illumination intensities, and a_f and $a_o(x)$ are illuminated area functions for the film and the a plane, perpendicular to the x-direction, in the aqueous phase, respectively. N_f is the number of ANS molecules per cm^2 of black film and N_o the number per cm^3 of the aqueous phase. The respective molecular extinction coefficients for the dye molecules in the film and aqueous phase have been assumed to be identical. As only their quotient will be needed later on, they have been omitted from Eqns 3 and 4. Should the values be different, it is easy to reintroduce them in the final stage of the analysis. L, the integrated light intensity in the illuminated layer of the aqueous phase, is given by

$$L = \int_{x_w}^{x_s} I_o(x) a_o(x) dx$$
$$= I_o(x_f) a_o(x_f) d = I_f a_f d \quad (5)$$

where x_s and x_w are indicated in Fig. 5, and d is the equivalent thickness of the illuminated region of the aqueous phase.
From Eqns 1–5

$$\Phi_f = \Phi_o d \frac{i_{f,max} S_f N_o}{i_{o,max} S_o N_F} \quad (6)$$

Strictly speaking, $I(x)$ should be replaced by $I(x, r)$, r being the radial coordinate in the illuminated plane, since the illumination cannot be assumed to be homogeneous in the plane defined by x. It is clear from Eqn 6, however, that owing to Eqn 5 this is unnecessary.

The quantities N_o, ϕ_o, S_f/S_o, d and N_F were either known already, or could be found by experiment. S_f/S_o was obtained by the graphical integration of the corrected spectra which had been appropriately scaled to be of equal i_{max}. Numerically, S_f/S_o was found to be 0.75. The equivalent thickness of the illuminated region of the aqueous phase, d, was determined in the manner suggested by Eqns 4 and 5 and without any knowledge of the absolute values of $I(x)$ and $a(x)$ (*cf.* Fig. 5). A thin film of a material which scattered light was positioned in a plane parallel to the black film, and was moved along the x-direction. The effective area A of this lamella was determined by the image field diaphragm of the instrument. The light intensity $I(x)a(x)$ as measured

by the photomultiplier, plotted in arbitrary units as a function of x, should show a maximum in the black film plane (Fig. 5). The integration of the curve between x_w and x_s and division of the result by $I_f a_f$ yields d. Experimentally, this was accomplished by the use of a thin Formvar film which contained numerous very small holes and which was freely suspended over a 4-nm hole in a thin brass sheet. (Similar films are widely used in electron microscopy.) A micromanipulator was used to move the film in the cell, the inner PTFE vessel having been removed. The result of the measurements is shown in Fig. 6, and d was calculated to be 0.54 mm. (It was confirmed during the course of these manipulations that the light intensity collected by the microscope did not, within certain limits, depend on the focal position of the objective.)

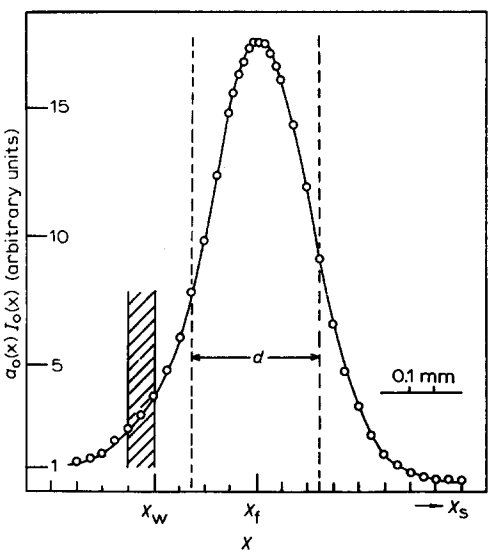

Fig. 6. The observed light intensity distribution in the black film cell according to the scheme of Fig. 5.

N_f was determined from the dependence of interfacial tension on ANS concentration (N_o) and the application of the Gibbs adsorption equation. It has been shown that, to a sufficiently good approximation, the tension of an equilibrium black lipid film of glyceryl monooleate and aliphatic hydrocarbon is equal to, and varies with surfactant concentration in the same way as twice the tension of the bulk lipid solution–aqueous phase interface[10,11,16]. N_f was thus taken to be twice the surface excess of ANS (Γ) at a single interface. The interfacial tensions for the single interface were determined by the drop volume method. As the ANS concentration did not exceed 10^{-3} M and as, below this concentration, Beer's law was obeyed, the activity coefficients for the ANS were taken as unity.

The results of the adsorption measurements are shown in Fig. 7. The data may be fitted with an equation of the Langmuir type in which the saturation adsorption parameter is $3 \cdot 10^{13}$ molecules/cm^2.

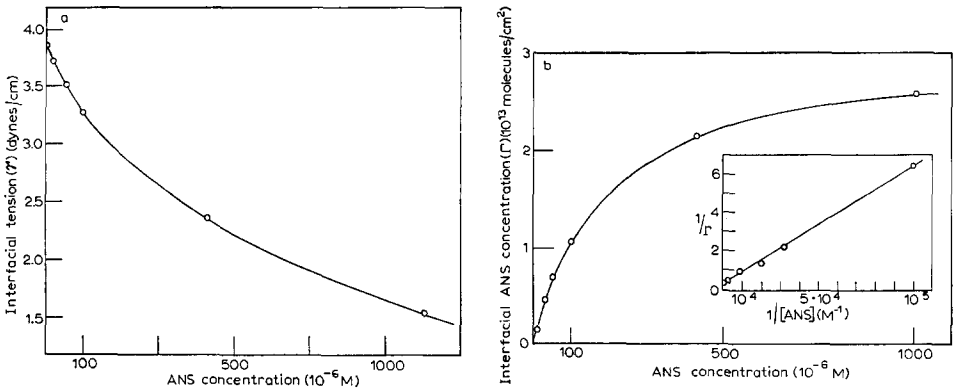

Fig. 7. (a) Tension of the *n*-decane water interface as a function of ANS concentration in the aqueous phase. The glyceryl monooleate concentration in the *n*-decane was approx. $7 \cdot 10^{-3}$ M. The aqueous phase was 0.1 M NaCl. (b) Interfacial concentration Γ of ANS molecules *vs* aqueous concentration, *c*. Γ was obtained from the data of (a) by means of the Gibbs equation. Inset: Langmuir plot of (b), $\Gamma_{max} = 3 \cdot 10^{13}$ molecules/cm².

Assuming a constant value of ϕ_f

$$i_{f,max} = k_f N_f \tag{7}$$

and

$$i_{o,max} = k_o N_o$$

With Eqn 6, these equations yield

$$\Phi_f = \Phi_o d \frac{k_f}{k_o} \cdot \frac{S_F}{S_o} \tag{8}$$

The constants k_o and k_f could be determined directly from graphs of i_{max} *versus* N.

For the aqueous phase the relevant plot is shown in Fig. 8a. At higher ANS concentrations a correction for light absorption in the aqueous phase (the inner filter effect) must be applied. This correction may be deduced from the known illumination geometry and the extinction coefficient of ANS in water, and gives results in good agreement with photocurrents obtained by linear extrapolation from low concentrations. The same correction factor has to be applied to the film fluorescence intensities. The corrected data are shown in Fig. 8b. It appears that the quantum yield is constant over the concentration range studied. The value of k_f/k_o was found to be $1.33 \cdot 10^3$ cm^{-1} so that, taking $\phi_o = 0.004$ (ref. 15),

$$\Phi_f = 0.004 \cdot 0.054 \cdot 1.33 \cdot 10^3 \cdot 0.75$$
$$= 0.22$$

No allowance has yet been made for a change in the molar extinction coefficient of the adsorbed ANS. This change could not be measured. It may, however, be estimated from the molar extinction coefficient of ANS in methanol where the corrected fluorescence peak is shifted to 490 nm. In methanol and water the molar extinction coefficients are $6.6 \cdot 10^3$ $moles^{-1} \cdot cm^{-1}$ and $5 \cdot 10^3$ $moles \cdot cm^{-1}$, respectively. On this basis an increase of approx. 10% may be assumed for the ANS adsorbed on to glyceryl

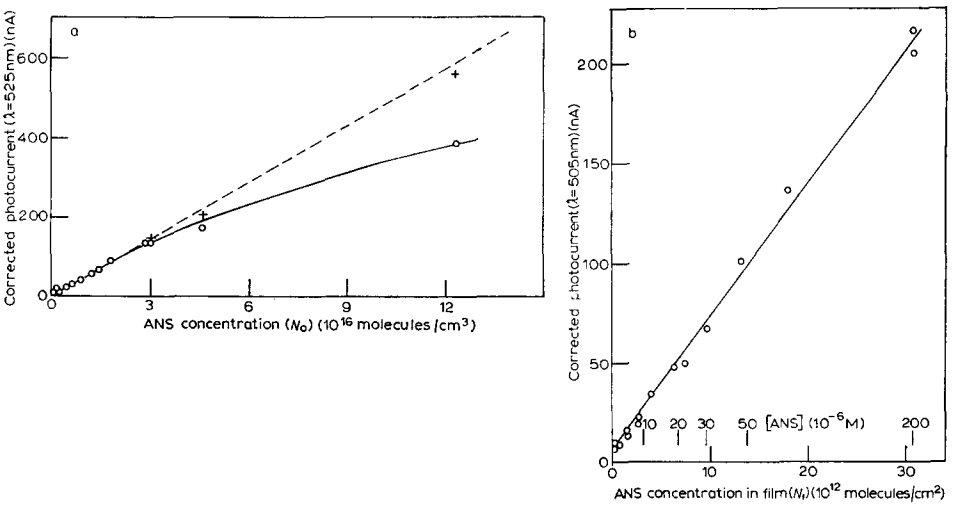

Fig. 8. (a) The corrected fluorescence intensity at $\lambda = 525$ nm (in units of photocurrent) of the aqueous phase *vs* ANS concentration (○—○). The correction for inner filter effects is done by extrapolation from low concentration values (broken line) or by estimation of the absorption from the extinction coefficient and the known illumination geometry (+--+). Each point represents a different filling of the cell with aqueous phase of the respective ANS concentration. (b) The corrected intensity of glyceryl monooleate film fluorescence *vs* number of ANS molecules per cm^2 of black film ($\lambda = 505$ nm) after correction for inner filter effects in the aqueous phase, using correction factors derived from (a). The quantum yield is constant (no concentration quenching in the range studied). Each point represents a different black film at the given ANS concentration.

monooleate membranes. From this reasoning $\phi_f \sim 0.2$. The overall accuracy of the determination leaves, at best, a 10% uncertainty in ϕ_f.

Sonicated lipid dispersions

Sonicated dispersions of egg lecithin (approx. 10^{-3} M in 10^{-1} M NaCl and approx. 10^{-5} M ANS) were put into the black film cell with the inner teflon cup removed. The wavelength of the fluorescence maximum was the same as that observed for black films ($\lambda_{max} = 492 \pm 3$ nm) of the lipid. Glyceryl monooleate dispersions prepared as described earlier were measured in the Fica 55 instrument and at a sufficiently high excess of lipid (concentration $\geqq 5 \cdot 10^{-4}$ M) showed a fluorescence emission spectrum, which was, within the spread of values of the black film measurements, identical in shape to that obtained from the latter system.

The quantum yield of the ANS in the glyceryl monooleate dispersions was determined relative to the known quantum yield in ethanol ($\phi = 0.37$)[15] by plotting the reciprocal values of the apparent quantum yields (as determined by integration of the spectra) *versus* the reciprocal nominal lipid concentrations. The latter varied between $5 \cdot 10^{-5}$ and 10^{-3} M. Before this was done, correction factors calculated to compensate for inner filter and light scattering effects (based on absorbance measurements) were applied. The reciprocal plot, which is a straight line for lipid concentrations $\geqq 2 \cdot 10^{-4}$ M was then extrapolated to infinite lipid concentration. This procedure resulted in a quantum yield of ANS in glyceryl monooleate of 0.17 ± 0.04. Extrapolation of the data to zero lipid concentration on a direct plot of apparent quantum yield *versus* lipid concentration yielded the known value for ANS in water[15], namely 0.004 ± 0.0005.

DISCUSSION

It must be pointed out that there is no difference between the fluorescence of coloured and black films. This may be contrasted with the findings for black films containing chlorophyll *a*, where the fluorescent chlorophyll has to be dissolved in the film-forming solution[5-7]. It is concluded that the membrane interior does not contain ANS in quantities detectable by the present technique. The amphipathic dye molecules are hence located predominantly at the surfaces of the membrane, as might be expected from surface chemical principles. A similar conclusion has recently been put forward on the basis of an X-ray diffraction study[17].

The position of the emission maximum of the fluorescence of ANS adsorbed on to black films is the same as that for aqueous dispersion of the lipids. For membranes of glyceryl monooleate the blue shift is smaller and, to interpret this, it is necessary to consider more than just the difference in the polar group. For example, the area per lipid molecule in glyceryl monooleate films is approx. 39 $Å^2$, whereas in phosphatidylcholine films the corresponding value is approx. 61 $Å^2$ (ref. 11). In the phospholipid membranes, therefore, there is likely to be a greater opportunity for the ANS molecules to interact with the hydrocarbon chains of the lipid and hence the polarity of the environment might well be lower in this instance. It has been shown elsewhere that the aliphatic hydrocarbon, the presence of which in the black film constitutes the essential difference between this structure and the leaflet of the liquid crystalline lipid, does not significantly affect the spacing of the polar groups of the lipid but serves principally to increase the membrane thickness[10,11]. The similar fluorescence behaviour of ANS in the two types of structure is consistent with this conclusion and confirms that the black film, if prepared and used correctly, can for certain purposes be a satisfactory substitute for a pure bimolecular leaflet[18]. It also points towards the further application of the technique to gain more information on the properties of the aqueous interfaces of monolayers and black films. These are relevant problems to the use of the latter system as a membrane model for transport studies[19].

There are at present no firm theoretical arguments with which to interrelate the nature of the environment and the quantum yield of ANS. Moreover, polarisation effects may be present as a consequence of a preferred chromophore orientation. This would have to be the subject of a detailed polarisation study which in principle is possible with the present technique.

Nevertheless, if one takes the quantum yield determined on the black film as a value which is not unreasonable in relation to the blue shift, then the apparent internal consistency of the combined spectroscopic and thermodynamic approach is reassuring in itself. The fact that a very similar value of the quantum yield was obtained by an independent method satisfactorily confirms the validity of the thermodynamic approach to the membrane composition *via* the interfacial tension measurements.

APPENDIX

Control behaviour and elimination of artifacts

The subtraction procedure is based on the assumption that the aqueous phase fluorescence is not changed by the formation of black films in the system. For glyceryl monooleate membranes this appeared to be so, the aqueous phase spectra recorded

before and after membrane formation being the same regardless of the number of films formed. For egg phosphatidylcholine membranes, however, the aqueous phase spectrum changed both in shape and in intensity during this process, there being a shift towards shorter wavelengths and an increased intensity after each film had been destroyed (see Fig. 4). This observation is explained by the fluorescence contributed by numerous small particles of lipid which are formed every time a film ruptures. The particles can be seen under dark field illumination in visible light and tend to be concentrated in the vicinity of the ruptured membranes. They can be removed readily with the aid of a Pasteur pipette, a procedure which remains effective for up to five to ten membranes.

Ultraviolet light scattered by the membrane itself may also bring about increased aqueous phase fluorescence. This effect is potentially serious because it cannot easily be separated from genuine membrane fluorescence. The scattering may be caused by dust particles, which are difficult to avoid with the brush technique, or the edge of the black film if the illuminated area is too big. Lenses of solvent trapped in the films[9], which have a wide range of sizes, also constitute an important source of scattered light. The large number of these lenses, which are often present, cause considerable light scattering, which may be measured when no ANS is present in the aqueous phase.

Only optically clean membranes, which may be obtained either by using certain solvents or by waiting for the lenses which are formed to reach and coalesce with the torus, yield a spectrum which is, in the absence of ANS, indistinguishable from the aqueous phase spectrum. Observation in visible light of the membranes under the same conditions of dark field illumination as used for the fluorescence excitation provided an excellent method for detecting the undesirable lenses and, in this respect, the use of a good microscope proved very beneficial.

As expected, the scattering caused by thick coloured films was always somewhat stronger than that of a black film. Moreover, the scattering decreased with time as the film drained. Within the limits of the spread of values caused by this effect, there was no significant difference between coloured and black films.

The spectra were independent of the film area as long as the latter was larger than the illuminated area. The spectra were also independent of the size of the image field diaphragm as long as this was larger than the illuminated field and smaller than the black membranes. A decrease in the size of the image field diaphragm caused merely a decrease in the intensities of the spectra. The aqueous phase spectra were independent of the presence or absence of the inner PTFE cup. It was concluded from these observations that the effects of the film edge and of the PTFE support had been effectively eliminated. In all experiments the cell was filled with 15 ml of aqueous solution, giving a level approx. 3 mm above the hole in the PTFE support. Withdrawal of up to 10 ml of aqueous phase (just short of emptying the hole) did not affect the aqueous phase spectra, indicating that only the small volume in the hole contributes to the measured fluorescence.

A small peak of irreproducible intensity was observed at about 450 nm for the aqueous phase, even when ANS was absent (Fig. 3). It was due to scattered ultraviolet light not completely suppressed by the filters and decreased slowly during the course of a few hours, suggesting that the light was scattered by the microscopic air bubbles and dust particles in the aqueous phase. No attempt was made to out-gas or filter the

solutions. Reliable spectra could always be obtained for ANS concentrations of approx. 10^{-6} M or more (see Fig. 3b).

The fluorescence intensities showed a small (5–10%) decrease during the first minutes, presumably owing to a photoreaction caused by the high illumination intensity. Blocking the path of the exciting light for a comparable length of time completely restored the fluorescence to its initial value. As the recording of a spectrum took about 1 min, this was always done after the fluorescence intensity had reached a constant value. By blocking the light path and by point-wise construction of the spectra it was established in a control experiment that the fading effect did not change the shape of the spectra. For the determination of the quantum yield, however, initial intensities at the wavelengths of the fluorescence peaks have been used.

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